

Communication

An Essential Histidine Residue in GTP Binding Domain of Bovine Brain Glutamate Dehydrogenase Isoproteins

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Greater than 90% of the original activity of the enzymes remained after modification of histidine residues of glutamate dehydrogenase (GDH) isoproteins from bovine brains with diethyl pyrocarbonate (DEPC). This suggests that the DEPC modified histidine residues are not critically involved in the catalysis of the GDH isoproteins. The influence of DEPC modified histidine residue(s) on binding of GTP to GDH isoproteins was investigated by protection studies. These studies showed that inhibition of GDH isoproteins by GTP was protected by preincubation of GDH isoproteins with DEPC. The amount of protection was dependent on the concentration of DEPC. The GTP inhibition was fully protected by preincubation of GDH isoproteins with DEPC at saturating concentrations. These results indicate that the histidine residues may play an important role in the GTP binding on GDH isoproteins. Spectrophotometric studies showed that three histidine residues per enzyme subunit were able to react with DEPC in the absence of GTP, whereas two histidine residues per enzyme subunit interacted with DEPC when the enzymes were preincubated with GTP. These results indicate that one of the histidine residues is involved in the GTP binding domain of GDH isoproteins. The quantitative affinity chromatographic studies showed that the influence of GTP on the binding of GDH isoproteins to DEPC-Sepharose was significantly distinct for the two GDH isoproteins. GDH I was more sensitively affected by GTP than GDH II in the binding affinity for

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DEPC-Sepharose. ADP, another well-known allosteric regulator, showed no significant changes in the interaction of DEPC with GDH isoproteins.

Keywords: Chemical Modification; Diethyl Pyrocarbonate; Glutamate Dehydrogenase; GTP Binding Domain; Reactive Histidine.

Introduction

Glutamate dehydrogenase (GDH) is found in nearly every organism and plays a pivotal role in nitrogen and carbon metabolism (Hudson and Daniel, 1993). GDH feeds the tricarboxylic acid cycle by converting L-glutamate to 2-oxoglutarate in the oxidative deamination reaction. It also supplies nitrogen for several biosynthetic pathways in the reductive amination reaction (Smith *et al.*, 1975). Recently, it was reported that the hyperinsulinism-hyperammonemia syndrome is caused by mutations in the GDH gene (Miki *et al.*, 2000; Stanley *et al.*, 1998). Infants with this disorder produce forms of GDH that are much less sensitive to inhibition by GTP, which demonstrates that the allosteric regulation of GDH plays a crucial role *in vivo*. It was proposed that this altered form of GDH would lead to higher oxidation rates due to increased levels of 2-oxoglutarate. This in turn would increase the ATP/ADP ratio in the pancreatic β cells that, via the ATP-induced closure of potassium channels, leads to the release of stored insulin granules.

The study of GDH is also of particular interest since the enzyme activity was altered in patients with neurodegenerative disorders that were characterized by multisystem

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Abbreviations: DEPC, diethyl pyrocarbonate ; GDH, glutamate

dehydrogenase.

atrophy and the predominant involvement of the cerebellum and its connections (Hussain *et al.*, 1989; Plaitakis *et al.*, 1984). The enzyme, isolated from one of the patients with a variant form of multisystem atrophy, displayed a marked reduction of one of the GDH isoproteins (Mavrothalassitis *et al.*, 1988; Plaitakis *et al.*, 1993). GDH is differentially distributed as various catalytically active isoforms of the enzyme (Abe *et al.*, 1992; Colon *et al.*, 1986; Plaitakis *et al.*, 1993). Although the origin of the GDH polymorphism is unknown, the presence of differently sized mRNAs, and multiple gene copies for human GDH, has been reported (Amuro *et al.*, 1988; Michaelidis *et al.*, 1993; Shashidharan *et al.*, 1994). Previously, Shashidharan *et al.* (1994) reported the presence of two human GDH-specific genes that encode highly homologous polypeptides. The one gene is expressed in all tissues, designated *GLUD1* (housekeeping GDH), whereas the other gene is expressed specifically in neural and testicular tissues, designated *GLUD2* (nerve tissue-specific GDH). According to their observations, the nerve tissue-specific GDH is relatively thermolabile. Recently, it was also reported that nerve tissue-specific, and housekeeping human GDHs, are regulated by distinct allosteric mechanism (Plaitakis *et al.*, 2000; Shashidharan *et al.*, 1997).

It is essential to have a detailed structural and functional description of the human GDH to elucidate the pathophysiological nature of the GDH related disorders. We isolated and characterized two types of GDH isoproteins (GDH I and GDH II) from bovine brains (Cho *et al.*, 1995; Choi *et al.*, 1999). Our work led to the finding that the two GDH isoproteins from bovine brains were differently regulated by ADP. Also, GDH I is relatively thermolabile, whereas GDH II shows a much longer half-life in heat-inactivation experiments (Cho *et al.*, 1995). We also identified several residues that are important for substrate and regulatory binding sites, and for catalysis using chemical modifications and photolabeling techniques (Ahn *et al.*, 1999; Cho and Yoon, 1999; Cho *et al.*, 1996; 1998; 1999; Kim *et al.*, 1997). In the present work, we report the involvement of a histidine residue in the GTP binding domain of brain GDH isoproteins by a combination of chemical modification and quantitative affinity chromatography.

Materials and Methods

Materials NADH, 2-oxoglutarate, glutamate, ADP, GTP, and DEPC were purchased from Sigma (USA). AH-activated Sepharose 4B was from Amersham Pharmacia Biotech (Sweden). Bovine brains were obtained from Majang Slaughterhouse (Korea). GDH isoproteins were purified from bovine brains by the method developed in our laboratory (Cho *et al.*, 1995). They

were homogeneous, as judged by a Coomassie-stained gradient SDS-polyacrylamide gel electrophoresis. All other chemicals and solvents were reagent grade or better.

Enzyme assay GDH activity was determined spectrophotometrically for the reductive animation of 2-oxoglutarate by measuring the decrease in absorbance at 340 nm, as described previously (Cho *et al.*, 1995), except that no ADP was used unless otherwise indicated. All of the assays were performed in triplicate. The initial velocity data were correlated with a standard assay mixture that contained 50 mM triethanolamine, pH 8.0, 100 mM ammonium acetate, 0.1 mM NADH, and 2.6 mM EDTA at 25°C. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 μ mol of NADH per min at 25°C.

Interaction of GDH with DEPC Modification of histidine residues in GDH isoproteins with DEPC was performed by the method of Ovadi *et al.* (1967). Briefly, a total of 500 μ g of enzyme was incubated at 25°C with various concentrations of DEPC in 25 mM HEPES, pH 6.0 in the presence and absence of 100 μ M GTP with a final volume of 1 ml. DEPC was dissolved in 95% ethanol. The final concentration of ethanol was kept below 5% during the incubation of the enzyme with the modifying agent. The DEPC concentration was diluted at least 10-fold during the assay for GDH activity. The formation of N-carbethoxyhistidyl was monitored at 240 nm with the extinction coefficient of 3200 M⁻¹cm⁻¹ at 240 nm (Ovadi *et al.*, 1967).

Quantitative affinity chromatography Affinity matrix, and quantitative affinity chromatography, was prepared by coupling of DEPC to AH-activated Sepharose 4B, as described by Veronese *et al.* (1979). Affinity chromatographic elutions were performed in the dark with a column of 1 \times 15 cm bed volume at 4°C. The 0.5 ml fractions were collected at a flow rate of 9 ml/h. GDH isoproteins (0.15 mg each) in an eluting buffer (20 mM potassium phosphate, pH 8.0 containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol) were applied to the column that was equilibrated with the eluting buffer that contained corresponding concentrations of DEPC. The elution position of GDH was determined by assays of fractions for GDH activity using NADH, as described previously. The variation of enzyme elution volume with DEPC was plotted according to the following equation, as previously reported by Veronese *et al.* (1979),

$$1/(V - V_o) = [L]K_{LM}/K_L(V_o - V_m)[LM] + K_{LM}/(V_o - V_m)[LM] \quad (\text{Eq. 1})$$

where V , protein elution volume; V_o , volume at which protein elutes in the absence of interaction; V_m , void volume as determined by Blue Dextran 2000; $[L]$, concentration of soluble ligand; $[LM]$, concentration of immobilized ligand; K_{LM} , dissociation constant for the immobilized ligand-protein interaction; and K_L , dissociation constant for the soluble ligand-protein interaction.

tion. K_L values were calculated from the ratio of ordinate intercept to the slope of $1/(V - V_o)$ vs $[L]$ plots.

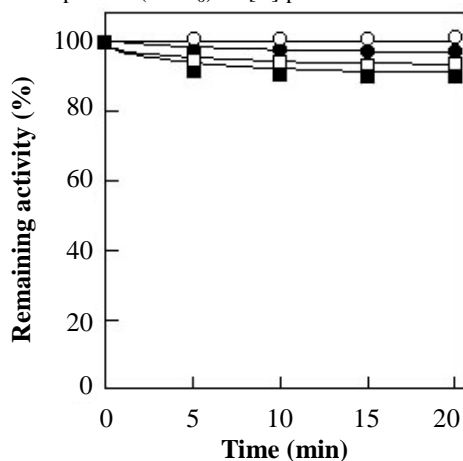


Fig. 1. Effects of DEPC on the activities of GDH isoproteins. DEPC was added to the GDH isoprotein solution in 25 mM HEPES, pH 6.0 at 25°C. At indicated times, the remaining activities were determined and expressed as a percentage of each control. All data represent mean values \pm standard deviation from three independent experiments. \circ , GDH I only; \square , GDH I + 100 μ M DEPC; \bullet , GDH II only; \blacksquare , GDH II + 100 μ M DEPC. Error bars are omitted for clarity.

Results and Discussion

In this study, we identified the involvement of a histidine residue in the GTP binding domain of brain GDH isoproteins by a combination of chemical modification and quantitative affinity chromatography. We used DEPC for the modification studies of histidine residues, because of its specificity. DEPC specifically reacts with histidine residues in proteins at pH 6.0 and yields an ethoxycarbonyl derivative with a characteristic absorption maximum at 240 nm (Pradel and Kassab, 1968). When the purified GDH isoproteins were incubated with DEPC up to 0.1 mM at 25°C, greater than 90% of the original activity of the GDH isoproteins remained (Fig. 1). These results suggest that the DEPC modified histidine residues are not critically involved in the catalysis of the GDH isoproteins. The influence of DEPC modified histidine residue(s) on the binding of GTP to GDH isoproteins was investigated by protection studies. The GDH isoproteins were preincubated with DEPC at various concentrations, and the aliquots of the mixtures were tested for GTP inhibition. The results in Fig. 2 show that the inhibition by GTP was protected by preincubation of the GDH isoproteins with DEPC. The amount of protection was dependent on the concentration of DEPC. The GTP inhibition was fully protected by preincubation of GDH isoproteins with DEPC at higher than 30 μ M (Fig. 2). These results indicate that the histidine residue(s) may

play an important role in the GTP binding on GDH isoproteins.

As estimated from a molar absorption coefficient of

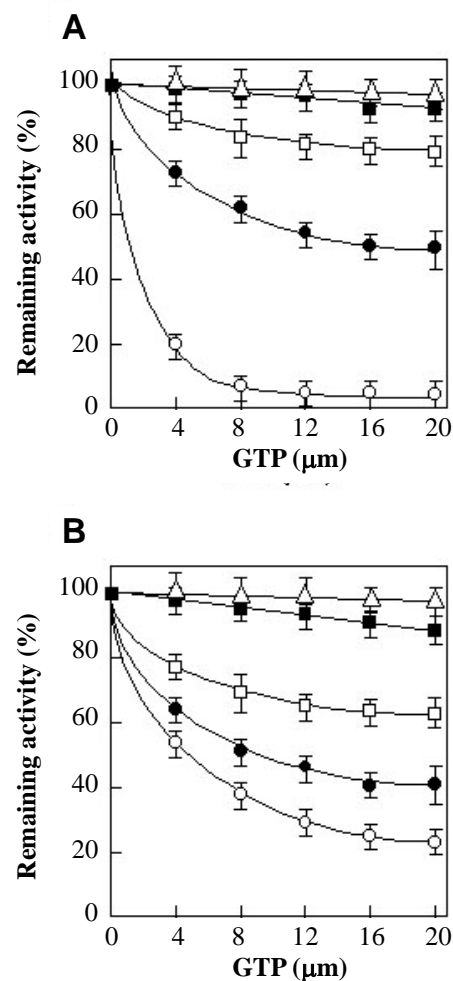


Fig. 2. Effects of preincubation of GDH I (A) and GDH II (B) with DEPC on the inhibition by GTP. Enzymes were preincubated with various concentrations of DEPC at 25°C in 25 mM HEPES, pH 6.0. Aliquots of the mixtures were assayed and the inhibition of GDH isoproteins by GTP at a final concentration of 10 μ M was measured. All data are expressed as a percentage of each control and represent mean values \pm standard deviation from three independent experiments. Final concentrations of DEPC were: \circ , 0 μ M; \bullet , 5 μ M; \square , 10 μ M; \blacksquare , 30 μ M; and \triangle , 50 μ M.

3200 $M^{-1}cm^{-1}$ at 240 nm, three histidine residues per enzyme subunit was able to react with DEPC in the absence of GTP, whereas two histidine residues per enzyme subunit interacted with DEPC when the enzymes were preincubated with 0.1 mM GTP (Table 1). The results indicate that one of the histidine residues is involved in the binding of GTP on GDH. In contrast to the effects of GTP, ADP showed no significant changes in the interaction of

DEPC with GDH isoproteins (Table 1). It was reported that DEPC might react under certain conditions, not only with the imidazole group, but also with tyrosyl residues **Table 1.** Effects of treatment of GDH isoproteins with GTP or ADP on the DEPC binding to the enzymes.

Treatment	[DEPC]/[subunit enzyme]	
	GDH I	GDH II
Enzyme only	0.1	0.1
Enzyme + DEPC	3.2	2.5
(Enzyme + GTP) + DEPC	1.9	1.9
(Enzyme + ADP) + DEPC	3.1	2.6

(Miles, 1977), and that the *O*-carbethoxylation of tyrosyl residues would result in a decrease of the difference absorbance at 278 nm (Burstin *et al.*, 1974). In the present study, absorption spectra that were taken in the near UV region (270–300 nm) are identical for both DEPC-reacted and intact enzymes (data not shown). This suggests that no modification of tyrosine has taken place.

To further investigate the effects of GTP on the binding of DEPC to GDH isoproteins, we undertook the direct characterization of the presumed binding processes by quantitative affinity chromatography on DEPC-Sepharose. The quantitative results with DEPC-Sepharose (shown in Fig. 3) provide further evidence for the regulatory property of GTP on the interaction of DEPC with GDH isoproteins. GTP was added in separate experiments to the eluting buffer at concentrations of 0.1 mM. The results in Fig. 3 show that a reduction of the elution volume of GDH isoproteins were observed. Also, their binding affinity was significantly changed in the presence of 0.1 mM GTP. The influence of GTP on the binding of GDH isoproteins to DEPC-Sepharose was significantly distinct for the two isoproteins. Figure 3 shows GDH I and the binding affinities for DEPC-Sepharose. The differences in the sensitivities to GTP between the two GDH isoproteins (Figs. 2 and 3) may be due to their micro-environmental structures. For instance, previous sequence data has suggested that residue 452 is Ala in GDH I, but Gly in GDH II (Cho *et al.*, 1996). The role of the Ala or Gly at the 452 position in the GTP binding to the GDH isoproteins remains to be studied. ADP, another well-known allosteric regulator of GDH, did not modify the elution volume of GDH isoproteins when added to the eluting buffer (data not shown). This suggests that competitive binding between ADP and DEPC does not occur. These results are consistent with those of protection studies (Table 1).

The enzyme activity of GDH is strictly regulated by allosteric regulators. Recent structural studies have proposed that most allosteric regulation is mediated by con-

trol of the mobility of the NAD^+ -binding domain. GTP inhibits the enzyme by promoting substrate binding, while ADP activates it by facilitating product release (Dieter *et*

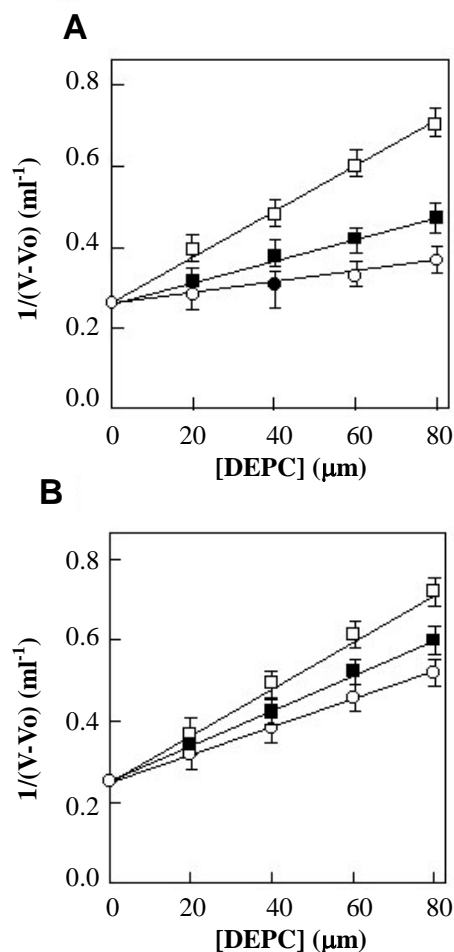


Fig. 3. Influence of GTP on the binding of GDH isoproteins to DEPC-Sepharose. $1/(V-V_0)$ against the total soluble DEPC concentration is plotted in the presence of varying amounts of GTP. Protein elution volume (V) was determined from the affinity chromatography elution profiles of GDH isoproteins on DEPC-Sepharose in the presence of varying concentrations of soluble DEPC in the eluting buffer. The concentrations of GTP used are: ○, 0 μM ; ■, 50 μM ; and ●, 100 μM . All data represent mean values \pm standard deviation for the three separate experiments. **A.** GDH I. **B.** GDH II.

al., 1980; Koberstein and Sund, 1973). When GTP binds to the allosteric site, the conformation of the NAD^+ -binding domain favors the closed position over the bound substrate or product. In contrast, ADP facilitates the opening of the active site cleft, thereby promoting substrate or product release. The importance of the allosteric regulations of GDH was further focused on recent reports of hyperinsulinism-hyperammonemia. It was reported that the hyperinsulinism-hyperammonemia syndrome is caused

by single mutations in the GDH gene that affects enzyme sensitivity to GTP-induced inhibition (Miki *et al.*, 2000; Stanley *et al.*, 1998). One of the mutations is a replacement of His450 with Tyr450. Recent atomic structures of bovine liver GDH (Smith *et al.*, 2001) indicate that His 450 (His454 in human sequence) lies on the pivot helix, and its side chain atom interacts with a β -phosphate of GTP. Therefore, the His450 mutation probably causes a steric interference with GTP binding. This suggests the importance of GTP and the regulation of GDH activity in the mammalian system. Although the precise His residue is not identified in the present work, it may be possible that His450 is responsible for DEPC modification and GTP binding. Further understanding of the mechanism and details of the regulation will help us to elucidate the metabolic role that GDH has in cellular homeostasis.

It was reported that the presence of two human GDH-specific genes encodes highly homologous polypeptides (Shashidharan *et al.*, 1994). The one gene, designated *GLUD1* (housekeeping GDH), is expressed in all tissues, whereas the other gene, designated *GLUD2* (nerve tissue-specific GDH), is expressed specifically in neural and testicular tissues. According to their observations, the nerve tissue-specific GDH is relatively thermolabile. Similar results have reported that bovine brain GDH I is relatively thermolabile. However, GDH II shows a much longer half-life in heat-inactivation experiments, and GDH I and GDH II are differently regulated by ADP and GTP (Cho *et al.*, 1995; 1996). Recently, it was also reported that nerve tissue-specific, and housekeeping human GDHs, are regulated by a distinct allosteric mechanism (Plaitakis *et al.*, 2000; Shashidharan *et al.*, 1997). The data in the present work show that the sensitivity of GDH I (relatively thermolabile form) to interaction with DEPC in the presence of GTP was significantly higher than that of GDH II (relatively thermostable form). These observations are consistent with previous reports that there are at least two different GDH activities that differ in their relative thermal stability and allosteric regulation characteristics (Abe *et al.*, 1992; Plaitakis *et al.*, 1984). These results suggest that the thermolabile GDH isotype has evolved into a more highly regulated enzyme. Also, the regulatory properties of GTP may be of importance for regulating glutamate fluxes *in vivo* under changing energy demands.

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